이학석사 학위논문

Identification of ACTL6A-mediated cell growth disruption in gastrointestinal cancer

ACTL6A가 위장암의 세포 성장에 미치는 역할 규명

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Abstract

Identification of ACTL6A-mediated cell growth disruption in gastrointestinal cancer

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The principle factors fundamental for gastric cancer (GC) development and outcomes are not well characterized resulting in a deficiency of validated therapeutic targets[1]. Previous studies have identified different subordinate groups of GC exhibiting not just genetic, but also distinct epigenetic hallmarks[2]. Accumulating evidence suggests that epigenetic peculiarities in GC are not mere bystanding events, but rather causative agents that advance tumorigenesis through active mechanisms[3].

Identification of the epigenetic modifiers that may participate in the proliferation of GC is the purpose of this study. To reach this goal, I analyzed the data from the GC cDNA microarray (GEO data) in the NCBI database to find the genes with selective GC growth-arrest. Subsequently, I performed CRISPR-Cas9 knockout screening in order to identify the epigenetic modifiers that affect GC cell growth. The analysis of the GEO data showed that Actlin-like 6A (ACTL6A) was up-regulated in GC patients, while the analysis of CRISPR screening indicated that depletion of ACTL6A decreased cell growth.

ACTL6A, a component of BAF(SWI/SNF) chromatin remodeling complexes, is important for cell differentiation. Nevertheless, its role and mechanism in GC has not been reported[4]. I investigated the functions and mechanisms of ACTL6A in GC proliferation. The knockout of ACTL6A (ACTL6A-KO) in 4 cell lines gradually decreased cell growth. I also found that the global transcriptional changes incurred following ACTL6A inactivation and the decrease of cell growth was caused by G2M arrest.

Considering that the same result occurred not only in the gastric cancer cells but also in the colon cancer cells, the inhibition of ACTL6A promotes decrease of cell growth in gastrointestinal cancer. Therefore, I identified ACTL6A as a candidate oncogenic driver in GC. The direct ACTL6A-regulated mechanisms its involvement in c ancer development will be uncovered through further study in progress. Thereby I will suggest ACTL6A as a noble prognostic or therapeutic target in gastrointestinal cancer.

Keywords : ACTL6A/ BAF53a/ Arp4/ INO80K, gastrointestinal cancer, epigenetics, cancer, oncogenic driver, CRISPR screen

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Introduction

GC is one of the most common cancer worldwide and the leading cause of cancer death. Currently available treatment options consists of surgical intervention, chemotherapy, immunogene therapy and target therapy[5]. The clinical outcome of GC mainly depends on the stage of tumor. Unfortunately, the median survival of GC patients is no more than 6~9 months[6]. Unrestricted proliferation of cancer cell and its ability of severe invasion and metastasis is the main cause of high malignancy degree and poorer patient survival. Even though the causal relationship between *Helicobacter pylori* and GC has been widely recognized[7][8], the molecular mechanisms, especially the epigenetic regulation, involved in the oncogenesis of GC remains largely unknown[9]. As a result, novel prognostic or therapeutic targets of GC can be developed through identification of epigenetic molecular proliferation mechanism.

Gene and thus protein expression are tightly controlled by the DNA sequence and are liable to irreversible mutational changes. However, the epigenetic state function independent of the DNA sequence, is heritable and reversible, and often has significant influence on gene expression. Therefore, in addition to a genetic influence, epigenetic dysregulations closely tie to the initiation, progression, and metastasis of cancer by influencing pathways that regulate cell division, cell

proliferation, and cell death[10]. Research over the past few decades has established that these epigenetic modifiers are associated with numerous diseases, especially cancer. Besides, with the involvement of epigenetics in cancer, these enzymes and protein domains provide new targets for cancer drug development. Therefore, these epigenetic modifiers can serve as suitable therapeutic strategies in cancer[11][12].

The lentiviral genome-scale CRISPR-Cas9 knockout (GeCKO) library was used to identify epigenetic modifiers essential for cell viability in cancer cells. The predominant method for genome-wide loss-of-function screens is RNA interference (RNAi), but its utility is limited by the confounding off-target effects and the inherent incompleteness of protein depletion[13]. The RNA-guided CRISPR (clustered regularly interspaced short palindromic repeats) Cas9 provides an efficient means of introducing targeted loss-of-function mutations at specific sites in the genome. Cas9 can be programmed to induce DNA double-strand breaks (DSBs) at specific genomic loci through a synthetic single-guide RNA (sgRNA), which can cause frame shift insertion/deletion (indel) mutations that result in a loss-of-function allele. Because the targeting specificity of Cas9 is presented by short guide sequences, which can be simply produced at large scale by array-based oligonucleotide library synthesis. The simplicity of CRISPR-Cas9 system to modify specific genomic loci proposes a new way to discover gene function on a genome-wide

scale[14].

Actin-like 6A (ACTL6A), known as BAF53a/ Arp4/ INO80K, is a subunit of SWI/SNF (BAF) complex. Previous reports revealed that ACTL6A was involved in various cellular processes, including chromatin remodeling, transcriptional regulation, vesicular transport and nuclear transition[15]~[20]. A recent study reported that ACTL6A is highly expressed in stem cells and progenitor cells enforcing the progenitor state by promoting cell self-renewal and preventing differentiation[21][22]. Increased ACTL6A expression has been reported in various cancers, including glioma, colon cancer, and osteosarcoma[23]~[25]. Another study found that ACTL6A interacts with oncoprotein c-Myc and has a role in c-Myc interacting nuclear complexes[26]. ACTL6A was also found to interact with TP63 and participated in the differentiation process in the epidermis and squamous cell carcinoma, including a Hippo signaling pathway regulator WWC1[27]. Nevertheless, biological role of ACTL6A in GC has not been reported.

In the current study, I examined ACTL6A expression in primary human gastric tissues and cell lines, and found that ACTL6A is overexpressed relative to normal gastric tissues and cell lines, and identified that depletion of ACTL6A decreased GC cell growth. Based on these results, the functional study was constructed to determine whether ACTL6A promotes GC progression, and to suggest that ACTL6A may represent a novel therapeutic target for GC.

Material and Methods

Cell culture

Cells were obtained from the Korea Cell Line Bank(AGS, MKN28, MKN74, SNU601, HCT116 and SW480) or American Tissue Culture Collection(CRL7869, CRL1459) and were not cultured for longer than two months. cells were grown in RPMI-1640 or DMEM supplemented with 10% fetal bovine serum (FBS;Welgene) and gentamicin(10μg/mL) at 37℃ in a humidified 5% CO2 atmosphere.

Acquisition and processing of public microarray data

Public microarray data was obtained from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo). The expression level of differentially expressed genes in gastric cells of patients and healthy donors was analyzed through The Cancer Genome Atlas (TCGA).

Establishment of patients-derived organoids and organoid culture

Human colon cancer tissues were obtained from Seoul National University Hospital (Seoul, Korea). The study protocol was approved by the Institute Review Board(IRB) of Seoul National University Hospital with informed consent (1608 - 054-784). Tumor cell isolation and organoid culture were processed as previously described[28].

Revers transcription PCR and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted using TRI reagent (Molecular Research Center) in accordance with the manufacturer's instructions. Total RNA(2μg) was reverse transcribed with random hexamers and ImProm-Ⅱ reverse transcriptase (Promega) as previously described[29].

Quantitiative real-time PCR (qRT-PCR) was performed by Step One Plus (applied Biosystems). cDNAs were amplified with SYBR Green (Molecular Probes) using Premix EX Taq (TaKaRa) as previously described[30].

Table1. Primer sequences for quantitative Real-Time PCR

GENE	SEQUENCE (5'→3')	
ACTL6A	F	CAGAGGCACCGTGGAATACT
	R	AGGACATAGCCATCGTGGAC
TRIM28	F	AGGACCATACTGTGCGCTCT
	R	GCAATGTTGCATGTTTGTCC
SSRP1	F	AAGCGGAAACAGCTCAAAAA
	R	GATGCCAGGATGGTCTGACT
NAT10	F	GTTCAGGAAAAGGCCATTGA
	R	GTCCATGCTCTTCAGCTTCC
PRMT1	F	AAGGAGCCCCTAGTGGATGT
	R	AAGGAGCCCCTAGTGGATGT
LGALS1	F	GACGCTAAGAGCTTCGTGCT
	R	GATGCACACCTCTGCAACAC
GPX8	F	TAATGGAGCTGCACTGGGAA
	R	CCTAAAGGGCAAGGGGAGAA

Virus production and transduction

The LentiCRISPRv2 vector system was obtained from Addgene (Cambridge, MA, USA, plasmid #52961) to deliver Cas9, sgRNA and a selective marker (puromycin) into target cells. Two distinct sgRNAs were designed for each genes to conduct double knockout using both at the same time. Lentiviruses were produced by transducing 293FT cells with lentiCRISPRv2 plasmids using Virapower packaging mix (Invitrogen) as previously described[30]. The viruses were harvested and the target cells were then incubated with viruses in the presence of 6μg/ml polybrene (Sigma). After a day, the transduced cells were cultured with 1μg/ml puromycin (Sigma) for another 6 days before harvest to select transduced cells.

For gene overexpression, the full-length human ACTL6A cDNA (pBS hBAF53a, Addgene, plasmid #17879) was cloned into MSCV PIG retroviral vector (Addgene, plasmid #18751) and transduced into target cells.

Expression change of target gene by using these viral vectors was validated by western analysis. The oligo sequences used for sgRNA are noted in Table 2.

Table 2. Oligo sequences used for sgRNA

GENE	SEQUENCE (5'→3')	
ACTL6A-4	F	caccg GTTGAAGGACATAGCCATCG
	R	aaac CGATGGCTATGTCCTTCAAC c
ACTL6A-5	F	caccgTGCCAAGACCTCGTAACCTG
	R	aaac CAGGTTACGAGGTCTTGGCA c
TRIM28-5	F	caccg CCTTGGTGTACTTCACCCGC
	R	aaac GCGGGTGAAGTACACCAAGG c
TRIM28-6	F	caccg TTGCACATAACCAGATCGCC
	R	aaac GGCGATCTGGTTATGTGCAA c
SSRP1-3	F	caccg TACAGAATGATGGTCGACTG
	R	aaac CAGTCGACCATCATTCTGTA c
SSRP1-4	F	caccg CGGCTCACCAAGAACATGTC
	R	aaac GACATGTTCTTGGTGAGCCG c
PRMT1-3	F	caccg TTTGACTCCTACGCACACTT
	R	aaac AAGTGTGCGTAGGAGTCAAA c
PRMT1-5	F	caccg GACTCACCCCGATGACCTTG
	R	aaac CAAGGTCATCGGGGTGAGTC c
NAT10-5	F	caccg TTCCACTGTTTCTACAGTCC
	R	aaac GGACTGTAGAAACAGTGGAA c
NAT10-6	F	Caccg AACTGAGGCCCATCAGGATG
	R	aaac CATCCTGATGGGCCTCAGTT c

Library construction and Preparation of gDNA for Next-Generation Sequencing

For the cloning of the targeted epigenetic modifiers library, I followed the custom sgRNA library cloning protocol as previously described[31]. I utilized the lentiGuide-Puro backbone(Addgene, plasmid #52963). I designed a targeted library to include epigenetic modifiers and control genes related with signaling of receptor tyrosine kinases. In total, I included 400 genes with 6 guides per gene. the pooled oligo library was ordered to match the vector backbone.

After cell selection and collection, genomic DNA was isolated from cell pellets using a genomic DNA isolation kit (Qiagen, #13343). For the genome-wide screen, after gDNA isolation, sgRNAs were amplified and barcoded as previously described[32]. PCR reaction consisted of 0.5μL of herculase2 fusion DNA polymerase, 10μL of herculase2 reaction buffer (Agilent, #600679), 1μg of gDNA, 1μL each of the 10nM primers, and water to 50μL total. The PCR cycling conditions were : 2 minutes at 95°C, followed by 20 s at 95°C, 20 s at 60°C, 30 s at 72°C, for 26 cycles; and a final 2 minutes extension at 72°C. After the PCR, all reactions were pooled for each sample.

Western blot analysis

Cells were washed twice with phosphate buffer saline (PBS). The cells were then harvested and incubated at 4°C for 30 min in a lysis buffer to which the protease, phosphatase and phosphatase inhibitor were added. Clear fluid was collected after centrifugation at 4°C for 30 min at 13,000 rpm. Protein concentration was determined through the BCA method. Later, the same amount of protein (1μg/lμl) obtained from each sample was run in a 10~12% SDS-PAGE gel and transferred onto a Nitrocellulose membrane. Blocking was done in 1% bovine serum albumin (BSA) and skimmed milk. and blots were incubated with the appropriate primary antibody at 4°C for over-night. This was followed by incubation with an HRP-conjugated secondary antibody for 1h at room temperature. Protein signals were detected using the enhanced chemiluminescent substrate (ECL).

ATP assay

Organoids were seeded in 96 well micro-assay-plates, at 4000 organoids /100μL/ well (n=3). ATP content was measured in accordance to the protocol of the CellTiter-Glo 3D cell viability assay kit (Promega #G9682).

Antibodies

Antibodies for the following were used in this study : PRMT1(sc166963), NAT10(sc271770), HA-probe (SC7392) was purchased from Santa Cruz Biotechnology; ACTL6A (A301-391A was purchased from bethyl laboratories; TRIM28 (ab10484) was purchased from Abcam; SSRP1(609701) was purchased from biolegend.

Colony formation assay

Knockout system transduced cells were seeded into six-well plates and cultured for 7 days. Seven days later, the media was removed and the cells were stained with Coomassie Brilliant Blue (Sigma) for 6 h at room temperature. Cells were washed two times in phosphate-buffered saline (PBS) and once in distilled water and air-dried. Colonies were counted using a GelCountTM automatic plate scanner (Oxford Optronix GelCount, Abingdon, UK) according to the manufacturer's instructions.

RESULTS

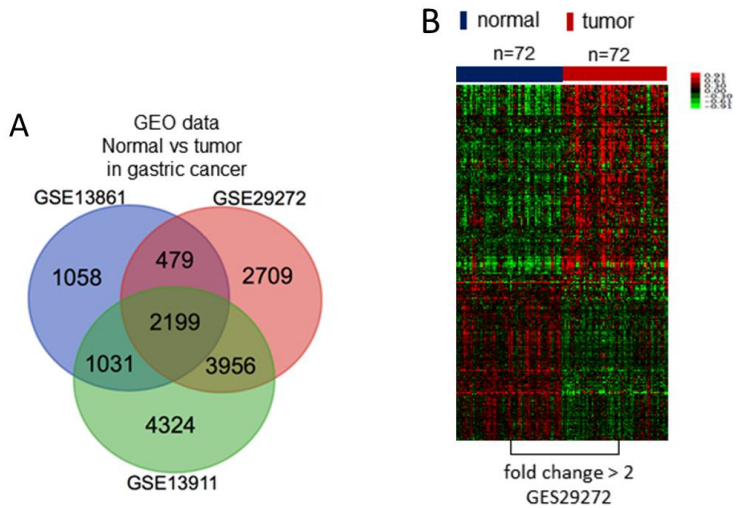
Identification of epigenetic modifiers important for GC growth

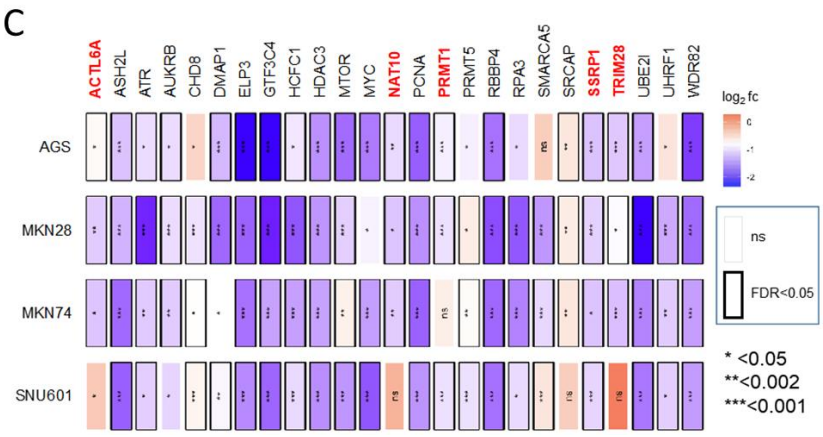
To screen for genes that play a important role in GC, I compared normal gastric samples with tumor samples by applying class comparison analysis using publicly available gene expression data (GSE13861, GSE29272, GSE13911)[1]. I identified 2,199 differentially expressed genes as being potentially cancer associated(Figures 1A and B).

Next, CRISPR-Cas9 knockout screening was assessed as a strategy for identifying essential epigenetic modifiers in GC cell growth. For this purpose, I employed a pooled library of 2,400 sgRNAs targeting 300 epigenetic modifiers, 100 control genes such as general transcription factors and receptor tyrosine kinases (six sgRNAs were designed for each gene) and performed positive and negative selection in 4 GC cell lines(AGS, MKN28, SNU601 and MKN74) by analyzing the effect of the gene expression on cell viability. The relative impact of each sgRNA on cell growth was assessed via Model-based Analysis of Genome-wide CRISPR-Cas9 Knockout (MAGeCK) method[33]. I ranked each genes based on its false discovery rate

(FDR) and revealed 25 genes as the top candidates, including mTOR and MYC known to play a role in many types of cancer such as GC (Figure 1C).

Among 25 genes, Five epigenetic modifiers (ACTL6A, TRIM28, SSRP1, NAT20 and PRMT1) overlapped with the 2,199 differentially expressed genes in tumor samples(Figure 1D). Therefore the five epigenetic modifiers that have not been previously studied in GC show selective GC growth-arrest.


A
GEO data
Normal vs tumor
in gastric cancer
GSE13861
GSE29272
GSE13911
1058
479
2709
2199
1031
3956
4324
B
normal
tumor
n=72
n=72
fold change > 2
GES29272


C
ACTL6A
ASH2L
ATR
AUKRB
CHD8
DMAP1
ELP3
GTF3C4
HCFC1
HDAC3
MTOR
MYC
NAT10
PCNA
PRMT1
PRMT5
RBBP4
RPA3
SMARCA5
SRCAP
SSRP1
TRIM28
UBE2I
UHRF1
WDR82
AGS
MKN28
MKN74
SNU601
log2 fc
ns
FDR<0.05
* <0.05
**<0.002
***<0.001

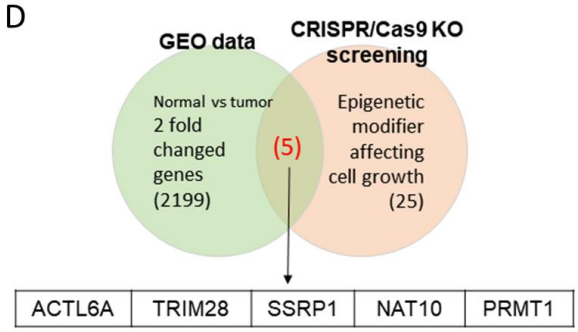

D
GEO data
Normal vs tumor
2 fold
changed
genes
(2199)
(5)
CRISPR/Cas9 KO
screening
Epigenetic
modifier
affecting
cell growth
(25)
ACTL6A
TRIM28
SSRP1
NAT10
PRMT1

Figure 1. Determination of epigenetic modifiers important for GC cells growth

(A) Venn diagram of genes showing significant differential expression between normal and tumor tissue in the three different GC patient cohorts. A univariate test (two-sample t-test) with a multivariate permutation test (10,000 random permutations) was employed. In each comparison, a cut-off p-value of less than 0.001 was applied to retain genes with an expression level that more than 2 fold differed significantly between the two groups of tissues examined.

(B) Expression patterns of selected genes shared in the three GC patient cohorts (GSE29272). The expression of 2,199 genes was commonly up- or down regulated in all three cohorts.

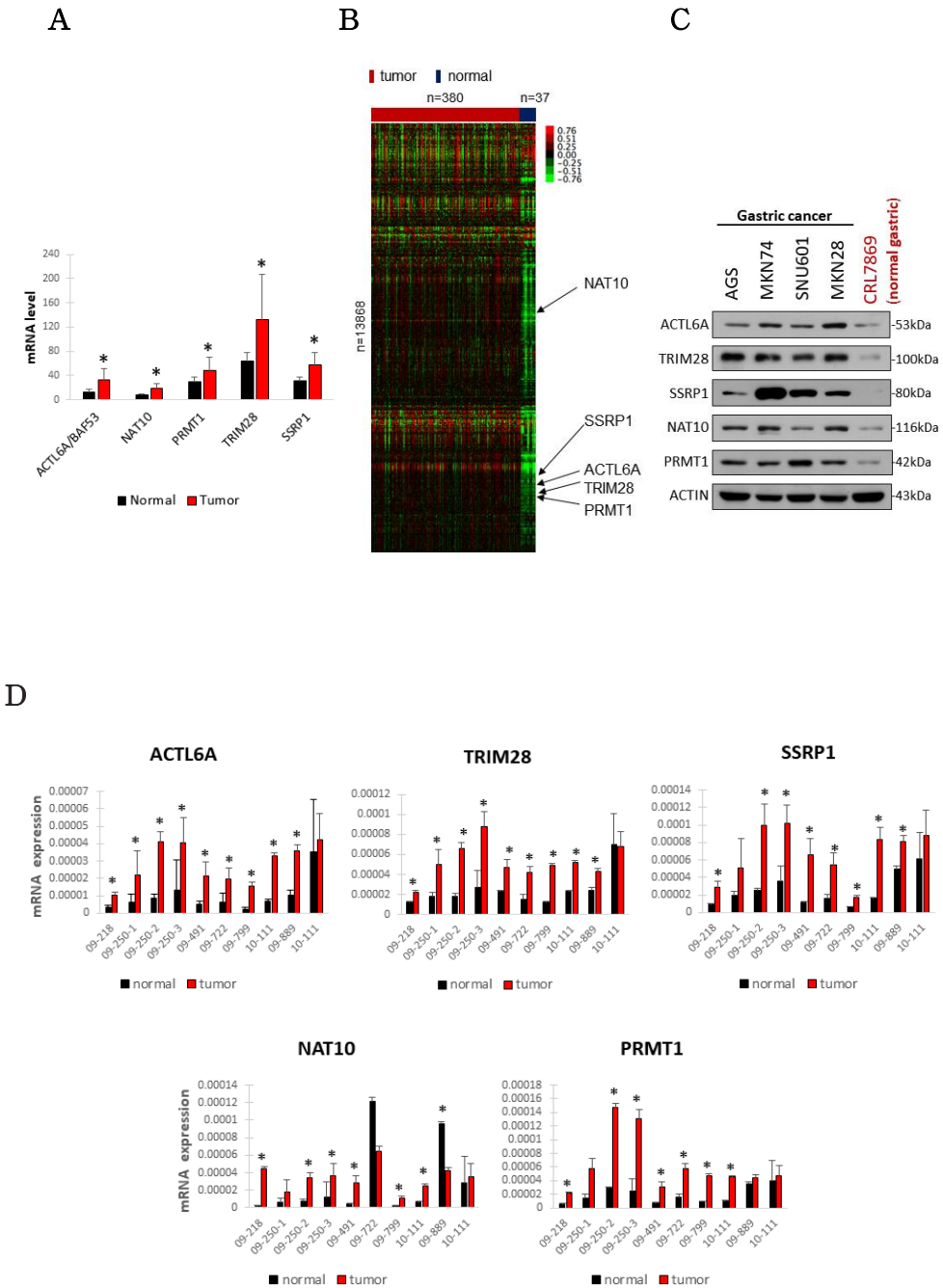
(C) Heatmap depicts the log2 fold-change of sgRNA abundance (averaging each independent sgRNA targeting a gene). Screening results were analyzed by using the MAGeCK algorithm. Raw data from different experiments are first normalized. Then, the statistical significance of each sgRNA is calculated by p-value. Essential genes are then identified by looking for genes whose sgRNAs are ranked consistently higher or lower. This is indicated by FDR. Significant genes have FDR<0.05 and p-value <0.05. * p-value<0.05, ** p-value<0.002, *** p-value<0.001.

(D) Venn diagram showing the overlapping genes between GEO data and CRISPR screening.

Five candidates are abnormally up-regulated in GC

Through two screenings (GEO screeing and CRISPR screeing), I found that sgRNAs targeting ACTL6A, SSRP1, NAT10, TRIM28 and NAT10 led to a potent and selective growth-arrest in GC. Hence, the screening results led me to hypothesize that these five genes act as epigenetic key molecules involved in GC cell growth.

To validate the results of the GEO screening, the expression levels of these five candidate genes between normal gastric and tumor GC tissues and cell lines were compared. The Cancer Genome Atlas (TCGA) data shows that the expression level of these genes is higher in tumor gastric tissues than that of normal gastric tissues(Figure 2A and B). Also, mRNA expression level in 10 pairs of gastric primary tissues is higher in tumor tissues than normal tissues(Figure 2D). I also found that protein and mRNA level of five candidate genes were higher in tumor cell lines (AGS, MKN28, SNU601 and MKN74) than normal gastric cell line (CRL7869)(Figures 2C and E). Taken together, I found that the expression level of five candidates was up-regulated in GC. This result suggest the possibility that these candidates may have oncogenic potential in GC.


A
B
C
D
mRNA level
ACTL6A/BAF53
NAT10
PRMT1
TRIM28
SSRP1
Normal
Tumor
tumor
normal
n=380
n=37
n=13868
NAT10
SSRP1
ACTL6A
TRIM28
PRMT1
Gastric cancer
AGS
MKN74
SNU601
MKN28
CRL7869
(normal gastric)
ACTL6A
TRIM28
SSRP1
NAT10
PRMT1
ACTIN
53kDa
100kDa
80kDa
116kDa
42kDa
43kDa
mRNA expression
normal
tumor

E

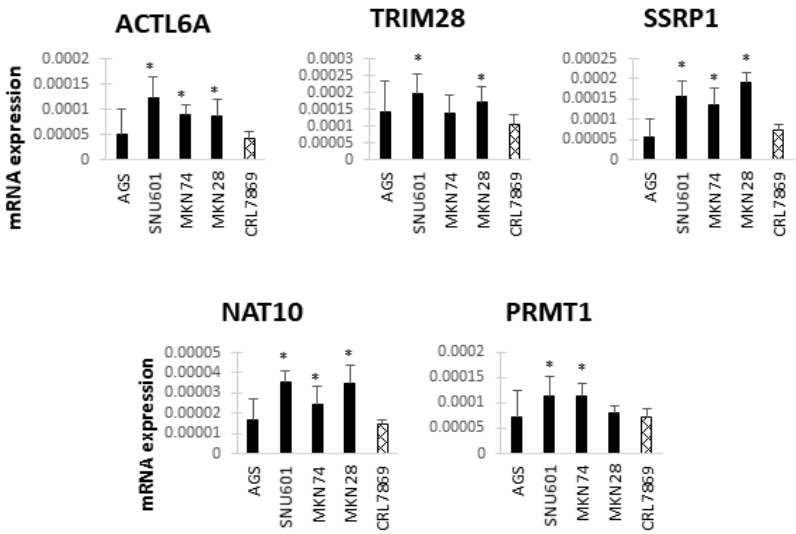


Figure 2. Expression levels of 5 candidates in GC patients and in cell lines

(A, B) Candidate genes expression in normal (n=37) and cancer tissues (n=380) from TCGA data.

(C) Western blot analysis of ACTL6A, TRIM28, SSRP1, NAT10 and PRMT1 from normal gastric cell line(CRL7869) and GC cell lines(AGS, MKN74, SNU601 and MKN28). Actin was used as a loading control.

(D) qRT-PCR analysis of ACTL6A, TRIM28, SSRP1, NAT10 and PRMT1 mRNA level in the indicated 10 pairs of normal and cancer primary gastric tissues. Relative mRNA levels for each gene were normalized to 18S levels.

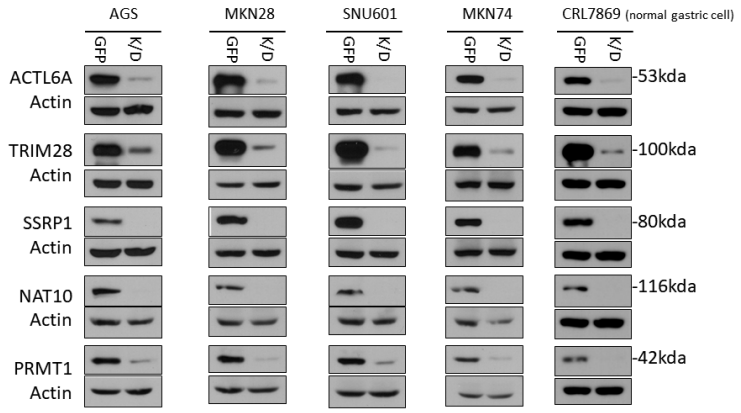
(E) qRT-PCR analysis of ACTL6A, TRIM28, SSRP1, NAT10 and PRMT1 mRNA level in the indicated cell lines.

Bars represent the mean ± SD of three independent experiments. *P < 0.05.

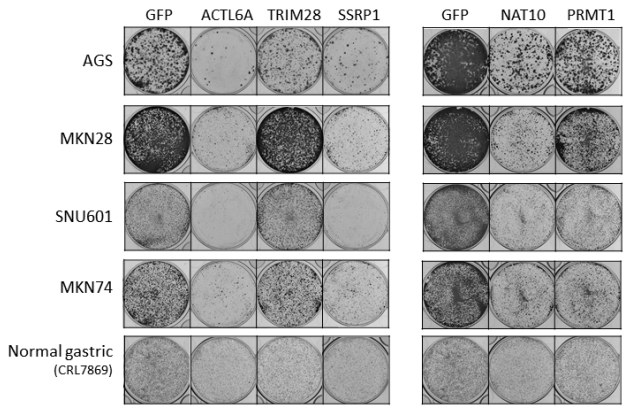
Depletion of the five candidates decreased cell growth in GC

To validate the results of the CRISPR screening, knockout of individual five candidates was performed in four GC cell lines used for screening and normal cell line as a control. The on-target effect of sgRNAs was verified by performing western blot assay(Figure 3A). Consistent with the results of the screening, targeting of ACTL6A, SSRP1 and NAT10 effectively impaired the cell growth of four GC cell lines(Figures 3B and D). However decrease in cell growth was less than 50% in normal cells(Figure 3C). There was no significant change of the cell growth in targeting TRIM28 and PRMT1(Figures 3B and D). Therefore, I found that ACTL6A, SSRP1 and NAT10 are essential for GC cell growth.

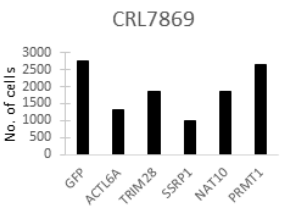
A


AGS
MKN28
SNU601
MKN74
CRL7869 (normal gastric cell)
GFP
K/D
ACTL6A
Actin
TRIM28
Actin
SSRP1
Actin
NAT10
Actin
PRMT1
Actin
-53kda
-100kda
-80kda
-116kda
-42kda

B


GFP
ACTL6A
TRIM28
SSRP1
GFP
NAT10
PRMT1
AGS
MKN28
SNU601
MKN74
Normal gastric
(CRL7869)

C


CRL7869
No. of cells
3000
2500
2000
1500
1000
500
0
GFP
ACTL6A
TRIM28
SSRP1
NAT10
PRMT1

D

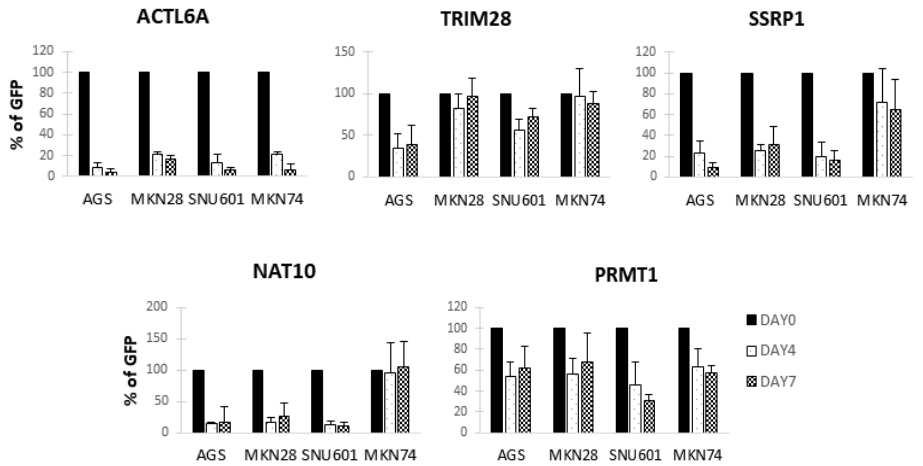


Figure 3. validation of the CRISPR screening results

(A) Western blot of five candidate genes in 4 gastric cells and normal cell transducing with the indicated sgRNAs. Cell extracts were prepared on day 6 or 7 post infection with the sgRNA. GFP was used as a negative control sgRNA. Actin was used as a loading control.

(B) Colony formation assay after depletion of the five candidate genes for 7 days.

(C) Quantitative analysis of colony numbers of CRL7869 in Figure 3B.

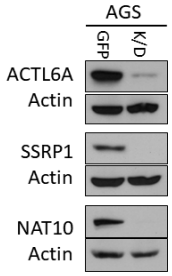
(D) Proliferation assay of individual sgRNAs performed in the indicated Cas9-expressing cell lines after day 7 of infection. GFP-targeting sgRNA expressing cells were considered as 100%. Bars represent the mean ± SD of three independent experiments.

Identification of ACTL6A as an essential transcription factor in GC

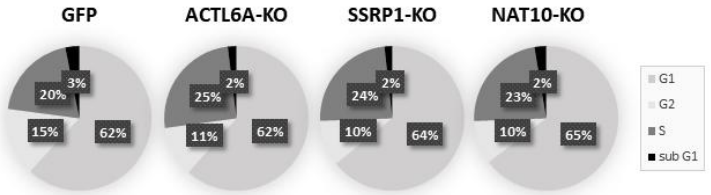
To evaluate a possible connection of ACTL6A, SSRP1 and NAT10 as essential transcription factors in GC, we performed whole genome transcriptome (WTS) to compare the global transcriptional changes after depletion of each genes. In these experiments, RNA was collected in sgRNA-transduced AGS immediately after the onset of the growth-arrest when the candidate genes were sufficiently reduced(Figure 4A). Surviving cells were collected at this time point to identify the mechanism regulated by the candidate, not what is normally observed from cell death(Figure 4B). The transcriptome results showed the number of genes signficantly altered after depletion of indicated candidate gene relative to the control(Figure 4C). Since ACTL6A regulated more genes than the others, I focused on ACTL6A to conduct further experiments.

To verify transcriptome result, I performed qRT-PCR with the most altered genes using three cell lines(AGS, MKN28 and MKN74)(Figure 4D). By identifing the gene set enrichment, I could predict that cell growth would decrease through G2M arrest after ACTL6A depletion(Figure 4E).

A



B

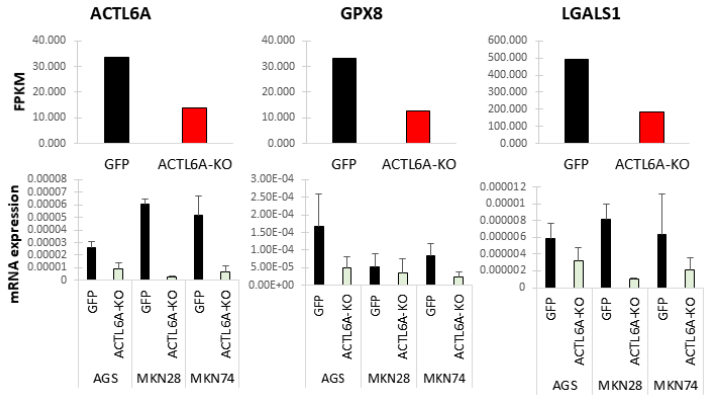


C

Result of WTS : number of genes regulated

GFP vs ACTL6A	333
GFP vs SSRP1	136
GFP vs NAT10	18

D



E

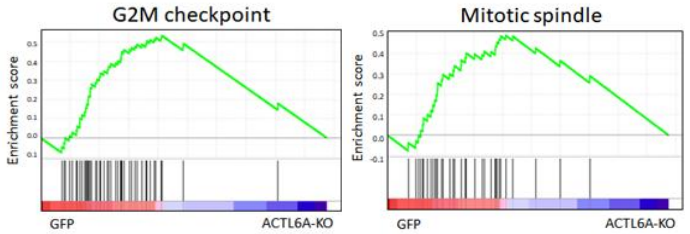


Figure 4. ACTL6A exhibits transcriptional regulation in GC

(A) Western blot of three candidate genes(ACTL6A, SSRP1 and NAT10) in AGS cell transduced with the indicated sgRNAs. Cell extracts were prepared on day 5 post infection with the sgRNA. Actin was used as a loading control.

(B) The cell cycle of surviving cells after transduction with the indicated sgRNAs. Cell extracts were prepared on day 5 post infection with the sgRNA.

(C) RNA-seq analysis of three candidate genes after depletion of each gene in AGS. Genes are ranked by p-value and fold change, compared with negative control(GFP). The number indicates significantly changed genes after depletion of candidate gene. (p-value < 0.05, log2 fold change >2)

(D) Validation of RNA-seq by qRT-PCR. The upper part is results of RNA-seq and the lower part is results of qRT-PCR. Three ACTL6A-depleted cells(AGS, MKN28 and MKN74) were used. Relative mRNA levels for each gene were normalized to 18S levels. Bars represent the mean ± SD of three independent experiments.

(E) In the enrichment plot, genes are ranked by signal/noise ratio according to their differential expression between GFP(negative control) and ACTL6A-KO(ACTL6A knockout). Genes in the gene set are marked with vertical bars, and the enrichment score is shown in green.

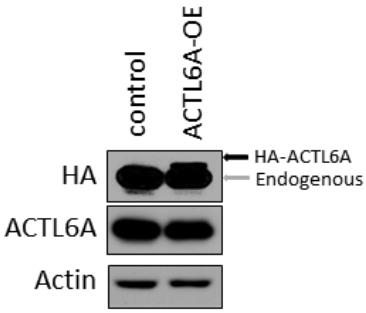
ACTL6A promotes tumorigenesis in GC

Finding that the depletion of ACTL6A reduced GC cell growth, the effect of ACTL6A on the tumorigenic activity of GC cells was evaluated. A knock-in transgenic AGS cell line was generated in which a hemagglutinin (HA) epitope tag are inserted with full-length human ACTL6A cDNA(Figure 5A). Establishment of stable overexpression was confirmed in protein and mRNA level(Figures 5B and C). The overexpression of ACTL6A significantly increased the growth rate of the CG cells(Figure 5D), and the colony forming assay showed that CG cells stably overexpressing ACTL6A formed more and larger colonies than the control cells (Figure 5E). These results indicate that overexpression of ACTL6A promotes the proliferation and tumorigenicity of GC cells.

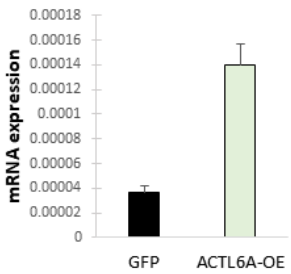
A


LTR
Puro R
IRES
GFP
LTH
HA
ACTL6A

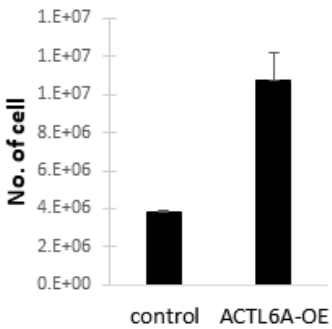
B


control
ACTL6A-OE
HA
ACTL6A
Actin
HA-ACTL6A
Endogenous

C


mRNA expression
0.00018
0.00016
0.00014
0.00012
0.0001
0.00008
0.00006
0.00004
0.00002
0
GFP
ACTL6A-OE

D


No. of cell
1.E+07
1.E+07
1.E+07
8.E+06
6.E+06
4.E+06
2.E+06
0.E+00
control
ACTL6A-OE

E

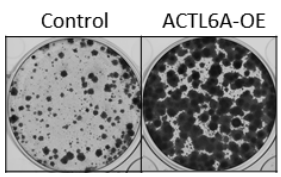

Control
ACTL6A-OE

Figure 5. ACTL6A overexpression results in increased proliferation of GC.

(A) Schematic diagram of HA-tagged ACTL6A overexpression (ACTL6A-OE) constructs. full-length human ACTL6A cDNA was cloned upstream from the IRES of the MSCV - puro-IRES - eGFP(MSCV-PIG) vector. The plasmid also contained EGFP and a puromycin resistance cassette. The backbone plasmid, MSCV-PIG, was used as a control.

(B) Western blotting analysis of HA-tagged and endogenous ACTL6A expression in AGS cell line. Actin was used as a loading control.

(C) qRT-PCR analysis of ACTL6A mRNA level in AGS cell line. Relative mRNA levels were normalized to 18S levels.

(D) Proliferation assay of stably ACTL6A-overexpressing AGS cell line for 7 days.

(E) Colony formation assay after overexpression of ACTL6A for 7 days.

Bars represent the mean ± SD of three independent experiments.

Candidate genes also up-regulated in colon cancer

To confirm that these findings extended to gastrointestinal, similar experiment was carried out using colon cells. Expression of three candidates(ACTL6A, SSRP1 and NAT10) were higher in colon adenocarcinoma tissues than normal colon tissues from TCGA data(Figure 6A). Also, protein level of three candidates were higher in nine tumor cell lines (CaCO2, DLD1, HCT8, HCT116, HT29, SW403, SW480, SNU1033 and LS174T) than normal colon cell line (CRL1459) (Figure 6B). Furthermore, two pairs of organoids derived from colorectal carcinoma patients demonstrated ACTL6A more expressed in tumor than normal(Figure 6C).

Among the three genes, further experiment was conducted for ACTL6A. Depletion of ACTL6A was performed in two colon cancer cell lines(HCT116 and SW480) and normal cell line(CRL1459). The on-target effect of sgRNAs was verified by performing western blot assay(Figure 6D). Consistent with the results of the GC, inactivation of ACTL6A effectively impaired the property of HCT116 and SW480 cells(Figures 6E and F). Even when the same experiment was performed on tumor organoid, the growth was decreased as the expression of ACTL6A decreased(Figures 6G, H and I). Therefore, I can conclude that ACTL6A is one of the essential epigenetic modifiers for gastrointestinal cancer cell growth.

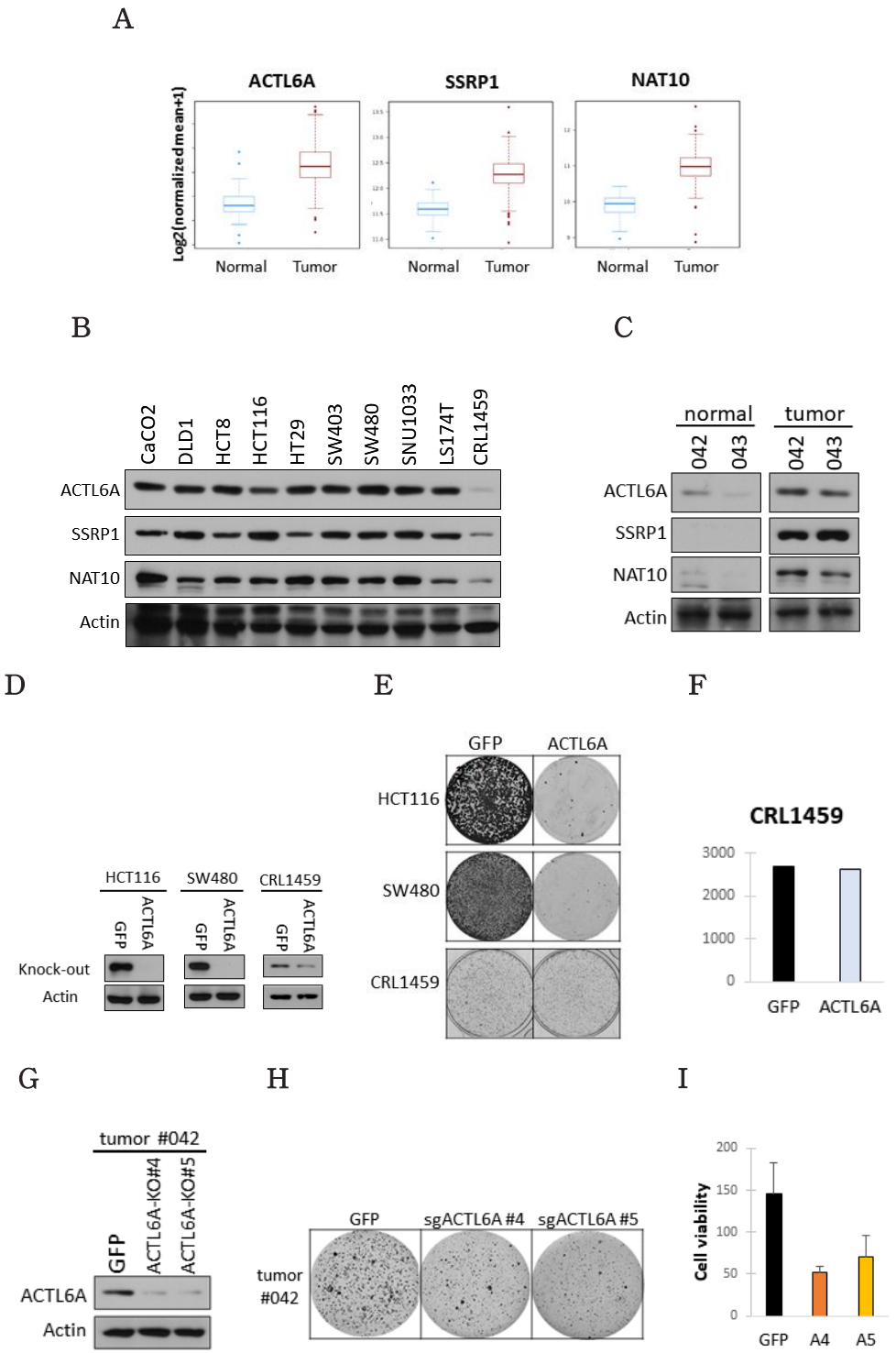

A
ACTL6A
SSRP1
NAT10
Log2(normalized mean+1)
Normal
Tumor
B
CaCO2
DLD1
HCT8
HCT116
HT29
SW403
SW480
SNU1033
LS174T
CRL1459
ACTL6A
SSRP1
NAT10
Actin
C
normal
tumor
042
043
042
043
ACTL6A
SSRP1
NAT10
Actin
D
HCT116
SW480
CRL1459
GFP
ACTL6A
Knock-out
Actin
E
GFP
ACTL6A
HCT116
SW480
CRL1459
F
CRL1459
3000
2000
1000
0
GFP
ACTL6A
G
tumor #042
GFP
ACTL6A-KO#4
ACTL6A-KO#5
ACTL6A
Actin
H
GFP
sgACTL6A #4
sgACTL6A #5
tumor #042
I
Cell viability
200
150
100
50
0
GFP
A4
A5

Figure 6. Decreased cell growth by reduction of ACTL6A in colon cancer

(A) Candidates expression in normal (n=41) and colon cancer tissues (n=262) from TCGA data.

(B)(C) Western blot analysis of ACTL6A, SSRP1 and NAT10 from colon cell line and two pairs of patient-derived organoid. Actin was used as a loading control.

(D) Western blot of three candidates in two colon cancer cell lines(HCT116 and SW480) and normal cell line (CRL1459) transducing with the indicated sgRNAs. Cell extracts were prepared on day 7 or 10 post infection with the sgRNA. GFP was used as a negative control sgRNA.

(E) Colony formation assay after depletion of the three candidates for 7 days in indicated cells.

(F) Quantitative analysis of colony numbers of CRL1459 in Figure 6E.

(G~I) Using two sgRNA individually, reduction of cell growth was confirmed after depletion of ACTL6A in tumor organoids.
(G) Western blot analysis with ACTL6A antibodies.

(H) Colony formation assay after depletion of ACTL6A for 7 days.

(I) Cell viability was measured by ATP assay.

Discussion

Here, I have revealed that ACTL6A have a potential as an oncogenic factor in gastrointestinal cancer. ACTL6A is a member of the SWI/SNF subunit of transcription factors(TFs) and is highly expressed specifically in stem cells and progenitor cells[16][17]. Previous reports have implicated abnormal up-regulation of ACTL6A in glioma, colon and osteosarcoma cancer[23]~[25], and the association of ACTL6A with various diseases. Furthermore, its overexpression are regulated by numerous genes related to cell differentiation or proliferation[34]. However, a detailed understanding of how ACTL6A contributes to cancer progression is still deficient.

In this study, abnormal up-regulation of ACTL6A was found in GC patients from GC GEO data sets. In addition, CRISPR-Cas9 knockout screening results confirmed that the reduction of ACTL6A caused decrease in GC cell growth. Therefore, I suppose that ACTL6A could have a role in GC proliferation. Depletion of ACTL6A alters the expression of genes involved in G2M checkpoint and mitotic spindle that could potentially contribute to its effects in GC. In order to determine whether ACTL6A functions as a trasncription factor that directly regulates oncogenic mechanisms, genome wide binding pattern analysis of ACTL6A is further required.

Oncogenes frequently have transcriptional change or mutation in human cancer, and oncogene activation leads to tumorigenesis[2].

Many TFs including APC, JUN, and SOX2 function as oncogenes[35], and up-regulation of TFs leads to tumorigenesis. Recent genomic analysis from public databases suggests that ACTL6A is not frequently demethylated or mutated in GC. Thus, the increased levels of ACTL6A in GC are likely a result of unconventional(non-canonical) oncogene TF mechanism. Thus, the mechanisms underlying the up-regulation of ACTL6A during GC progression will require further elucidation.

In summary, I have demonstrated that ACTL6A is one of novel oncogenic drivers in gastrointestinal cancer. These results propose that pharmacological inhibitors of ACTL6A may have a potential for therapeutic treatment of gastrointestinal cancer.

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국문 초록

위암의 발병과 결과의 근본적인 원인은 잘 규명되어 있지 않기 때문에 치료에 사용할 수 있는 표적이 부족하다. 이전의 연구에 의하면 위암의 여러 하위 그룹이 유전적 요인뿐만 아니라 후성유전적 특성을 나타내는 것으로 밝혀졌다. 또한 그동안의 연구에 따르면 위암에서의 후성유전학적 이상은 더 이상 부수적인 영향이 아닌 능동적인 기전을 통해서 발암을 촉진하는 것으로 밝혀졌다.

위암의 성장 및 증식에 관여하는 후성유전학 변형 인자를 확인하기 위해 NCBI 데이터베이스에서 위암의 cDNA 마이크로어레이 데이터(GEO 데이터)를 분석하여 정상보다 위암에서 더욱 특이적으로 영향을 미치는 유전자를 찾았다. 이 중에서 위암의 세포 성장에 영향을 미치는 후성유전학적 변형 인자를 확인하기 위해 CRISPR-Cas9 knockout 스크리닝을 진행했다. 이 두 가지 실험에서 중복되는 유전자로 Actin-like 6A (ACTL6A)를 발견하여, ACTL6A의 감소에 의해서 위암의 세포 성장 특이적으로 감소한다는 것을 밝혔다. 따라서 ACTL6A를 위암에서의 종양 유발 인자 후보로서 확인했다.

ACTL6A는 BAF (SWI/SNF) chromatin remodeling 복합체의 구성 요소이며, 세포 분화에 중요한 역할을 한다고 알려져 있다. 그럼에도 불구하고 위암에서의 역할과 이것이 관여하는 메커니즘에 대해서 보고된 적이 없다. 그래서 본 논문에서 ACTL6A가 위암의 성장 및 증식에 어떠한 역할을 하는지 조사하고자 한다. 실제로 CRISPR 스크리닝에서 사용된 4가지 위암 세포주(AGS, MKN28, SNU601, MKN74)에서 ACTL6A의 감소가 세포 성장을 감소시키는 것을 확인했다. 또한, ACTL6A의 불활성화에 따른 유전체 수준의 유전자 발현 변화를 확인한 결과, ACTL6A에

의한 세포 성장 감소가 G2M arrest에 의해 나타난다는 것을 확인했다.

이러한 결과가 위암에서뿐만 아니라 결장암 세포에서도 발생한다는 것을 확인하여서, ACTL6A의 억제는 위장 암에서 세포 성장의 감소를 촉진한다는 것을 입증하였다. 앞으로의 실험을 통해서 ACTL6A에 의해서 직접적으로 조절되는 메커니즘과 이것이 암 발생에 어떻게 관여하는지는 규명하여 ACTL6A를 위장 암 진단 또는 치료의 표적으로 제시하고자 한다.

주요어 : ACTL6A/ BAF53a/ Arp4/ INO80K, 위장암, 후성유전학, 암, 발암 유발 인자, CRISPR screen

학 번 : 2017-21481